DESCRIPTION

MARKER PROTEINS FOR DIAGNOSING LIVER DISEASE AND METHOD

OF DIAGNOSING LIVER DISEASE USING THE SAME

TECHNICAL FIELD

The present invention relates to a plurality
of serum proteins which have been found to increase or
decrease with the habit of drinking and to be hence
5   utilizable as marker proteins for diagnosing liver
disease, as a result of proteome analysis of serum
samples using the protein chip technology; and a method
for diagnosing the probability of the onset of a liver
disease, the liver disease, the prognosis of the liver
10   disease, or the like in, for example, a problem
drinker, by detecting or quantifying the above-
mentioned proteins.

BACKGROUND ART

15   The first step to the diagnosis of an organ
trouble caused by alcohol is to know an accurate
drinking history, but alcohol dependence is called a
denying disease, namely, an inveterate drinker does not
accurately report his (or her) alcohol intake in all
20   ages and countries. Therefore, an objective marker for
substantiating the alcohol intake is necessary. A
marker for the habit of drinking most commonly measured
is γ-GTP (GGT). However, even if a drinker has a high

GGT level, the GGT level does not always correlate with the degree of seriousness of liver trouble or the cumulative alcohol intake of the drinker. In addition, the change of GGT level after alcohol drinking is

5 dependent on individuals and there are a considerable number of so-called nonresponders who show no GGT increase even after drinking a large amount of alcohol.

On the other hand, by a cause other than drinking, GGT is often increased also in a person

10 having no drinking habit, such as a person having fatty liver due to fatness or a person who habitually takes a certain medicine. Guidance is often given, for example, in a hospital for complete physical examination so that a person having a high GGT level

15 may be hastily judged to be a drinker. Therefore, chain-deficient transferrin (CDT) has been developed by investigators in north Europe for examination complementary to the examination with GGT, and its usefulness is emphasized in European and American

20 references. But, in the case of the result obtained for Japanese, CDT as a marker of drinking permits detection of only about 10% of the GGT nonresponders.

Acetaldehyde, the first metabolite of ethanol is so reactive that it forms various acetaldehyde

25 addicts of various proteins. For example, attempts have been made to detect an acetaldehyde-hemoglobin addict by HPLC or the like. There is also an addict that is expected to be an interesting marker capable of

permitting estimation of alcohol intake in the past, such as HbAlc in the case of diabetes. But, such an addict has not been put to practical use because of its low sensitivity.

5   The habit of drinking is one of two major causes of chronic liver troubles. As to liver cirrhosis cases in Japan, the percentage of cases due to only alcohol itself is considered to be only 10 to 15%. This, however, is data obtained mainly in university hospitals and the like. Considering the presence of alcoholics in a number estimated at more than 2,000,000, it is speculated that there are many latent patients with alcoholic liver trouble who have no chance to get a medical examination in a medical institution. In addition, since the habit of drinking is a factor of the exacerbation of cerebral hemorrhage, hypertension, gout and the like, early and accurate screening of problem drinkers is very important. However, as described above, there is no marker having decisive sensitivity and specificity among existing so-called markers of drinking, and hence searching for a novel marker is desired.

        A general method for comprehensive expression protein analysis is two-dimensional protein electrophoresis, but this method is disadvantageous in the detection of low-molecular weight proteins or peptides. In recent years, a protein chip technology comprising a combination of surface enhanced laser

desorption ionization (SELDI) and a time-of-flight mass
spectrometer has been developed by Ciphergen Biosystems
Inc., USA and has been begun to be clinically used for,
for example, detecting a novel tumor marker.

5   Therefore, comprehensive search for a novel marker by
the utilization of such a proteomics technique and the
like is desired.

DISCLOSURE OF THE INVENTION

10   The present invention is intended to find a
novel marker capable of permitting early and accurate
screening of the liver diseases of problem drinkers or
the like, establish a system for measuring the marker
and take advantage of the marker in medical treatment.

15   The present inventors have done intensive
research on the above problem and consequently have
accomplished the present invention. That is, the
present inventors have succeeded in identifying novel
serum proteins each of which shows an increase or a

20   decrease with the habit of drinking, by the use of
serum samples periodically collected from alcoholics
hospitalized for giving up drinking. The present
inventors have found that these serum proteins are
utilizable as marker proteins for diagnosing liver

25   disease, whereby the present invention has been
accomplished.

Accordingly, the present invention relates to
a marker protein for diagnosing liver disease selected

from a protein which is a human fibrinogen α-E chain
decomposition product and has a molecular weight of
5,900 (5.9 kDa protein), a protein which is an
apolipoprotein AII decomposition product and has a
5   molecular weight of 7,800 (7.8 kDa protein), a protein
which is apolipoprotein AI and has a molecular weight
of 28,000 and variants of these proteins which have the
same function as that of the proteins as a marker
protein for diagnosing liver disease.

10   In addition, the present invention relates to
a method for diagnosing the probability of the onset of
a liver disease, the liver disease or the prognosis of
the liver disease by detecting or quantifying any of
the above-mentioned marker proteins for diagnosing
15   liver disease in a sample obtained from a patient who
is suspected to have the liver disease.

          Further, the present invention relates to a
novel protein having the amino acid sequence shown as
SEQ ID NO: 1 in the sequence listing, or its variant
20   having the same function as that of said protein as a
marker protein for diagnosing liver disease, which
variant is a protein having 90% or more homology with
said amino acid sequence or a protein having an amino
acid sequence formed by deletion, substitution or
25   addition of one or more amino acid residues in the
amino acid sequence shown as SEQ ID NO: 1.

          Still further, the present invention relates
to a novel protein having the amino acid sequence shown

as SEQ ID NO: 2 in the sequence listing, or its variant having the same function as that of said protein as a marker protein for diagnosing liver disease, which variant is a protein having 90% or more homology with

5 said amino acid sequence or a protein having an amino acid sequence formed by deletion, substitution or addition of one or more amino acid residues in the amino acid sequence shown as SEQ ID NO: 2.

Still further, the present invention relates

10 to a method for measuring any of the above-mentioned proteins or their variants by immunoassay by the use of an antibody against the protein or variant to be measured.

15 BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 shows the measurement results obtained from the serum of a patient with alcoholic liver trouble by the use of SAXII chips by utilizing Protein Chip System available from Ciphergen Biosystems Inc.

20 From the decrease in height of the peak, it can be seen that the level of the 28 kDa protein (apolipoprotein AI) decreases in the serum gradually with the lapse of time in the order of the level at the time of admission, the level after hospitalization for 1 week

25 and the level after hospitalization for 3 months.

Fig. 2 shows the measurement results obtained from the serum of a patient with alcoholic liver trouble by the use of WCXII chips in the same manner as

in Fig. 1. It can be seen that the blood levels of (1) the 5.9 kDa protein and (2) the 7.8 kDa protein increase with treatment with the lapse of time from the blood levels at the time of admission.

5   Fig. 3 shows the measurement results obtained from the serum of a healthy person by the use of WCXII chips in the same manner as in Fig. 1. The blood levels of (1) the 5.9 kDa protein and (2) the 7.8 kDa protein are definite high levels, and comparison

10   between Fig. 2 and Fig. 3 indicates that these proteins are decreased by the disease.

          Fig. 4 shows the result of electrophoresis of the 7.8 kDa protein and the 28 kDa protein by SDS-PAGE. It can be seen that the samples contain the proteins of

15   interest, respectively.

          Fig. 5 shows the mass spectrometry value and HPLC data of synthetic 5.9 kDa protein. It can be seen that the mass spectrometry value agrees with the theoretical value.

20   Fig. 6 shows the result of comparing data for the synthetic 5.9 kDa protein with data for the serum of a patient who does not drink, by the use of WCXII protein chip arrays by utilizing Protein Chip System available from Ciphergen Biosystems Inc. Both of them

25   show a peak at 5.9 kDa and they show the same behavior.

          Fig. 7 shows the result of measuring the height of a peak at 5.9 kDa in the case of a serum sample from each of healthy persons and patients, who

are various in alcohol intake, by the use of WCXII
protein chip arrays by utilizing Protein Chip System
available from Ciphergen Biosystems Inc. It was found
that the height of the peak decreases depending on the
5 alcohol intake.

Fig. 8 shows the result of measuring
absorbance by the use of an EIA method (a sandwich
ELISA method) by using samples containing various
concentrations of the 5.9 kDa protein. The axis of
10 abscissa refers to the concentration of the 5.9 kDa
protein and the axis of ordinate to absorbance
measured. The absorbance increases depending on the
concentration of the 5.9 kDa protein. That is, this
result indicates that the concentration of the 5.9 kDa
15 protein in the samples may be measured by the EIA
method.

MODE FOR CARRYING OUT THE INVENTION

The present invention is explained below in
20 further detail.

The proteins that have been found to be
utilizable as marker proteins for diagnosing liver
disease by the present invention are a protein which is
a human fibrinogen α-E chain decomposition product and
25 has a molecular weight of 5,900 (hereinafter referred
to as the 5.9 kDa protein), a protein which is an
apolipoprotein AII decomposition product and has a
molecular weight of 7,800 (hereinafter referred to as

the 7.8 kDa protein), and a protein which is apolipoprotein AI and has a molecular weight of 28,000 (hereinafter referred to as the 28 kDa protein). The 5.9 kDa protein, the 7.8 kDa protein and the 28 kDa

5 protein are proteins having the amino acid sequences shown as SEQ ID NOs: 1, 2 and 3, respectively, in the sequence listing.

The individual proteins are explained below. The 5.9 kDa protein is a human fibrinogen α-E chain

10 decomposition product, comprises 54 amino acids and has a theoretical molecular weight of 5904.2. The 7.8 kDa protein is a human apolipoprotein AII decomposition product, comprises 68 amino acids and has a theoretical molecular weight of 7753.8. The 28 kDa protein is

15 apolipoprotein AI, comprises 243 amino acids and has a theoretical molecular weight of 28078.8. As to the 5.9 kDa protein and the 7.8 kDa protein among the above proteins, the present invention has elucidated their presence in blood and their clinical significance in

20 liver troubles and the like for the first time. The 5.9 kDa protein and the 7.8 kDa protein are novel proteins and novel marker substances. Apolipoprotein AI, the 28 kDa protein is a known protein, its clinical significance in lipid metabolism has been established,

25 and its clinical measurement has heretofore been carried out.

The 5.9 kDa protein, 7.8 kDa protein and 28 kDa protein, which have been found to function as

marker proteins for diagnosing liver disease by the
present invention, are not limited to proteins having
the amino acid sequences shown in the sequence listing
but may be variants of these proteins which similarly

5   function as marker proteins for diagnosing liver
disease. That is, it should be sufficiently considered
that the 5.9 kDa protein, 7.8 kDa protein and 28 kDa
protein are very liable to be degraded by various endo-
and exoproteases particularly in blood and tissues,

10   resulting in changes of the total length of amino acids
and the length of the sequence. In producing a
recombinant protein, an amino acid variation capable of
changing the antigenicity as slightly as possible
should, of course, be given in order not to decrease

15   the efficiency of expression. Therefore, there may
also be used variants of the 5.9 kDa protein, 7.8 kDa
protein and 28 kDa protein of the present invention
which are proteins having 90% or more homology with the
amino acid sequence of each of the proteins of the

20   present invention (here, the term "homology" means the
sameness of amino acids). The amino acid sequence of
any of the variants may have a length changed by 15% or
less, and the present invention also includes such a
variant when it functions as a marker protein for

25   diagnosing liver disease. The variants are preferably
proteins having 95% or more homology, more preferably
98% or more homology. The homology of the amino acid
sequence may be looked up in well-known software, for

example, software obtained by adopting as a principle

the reference method described in the method of Wilber,

W.J., Lipman, D.J., et al. (Proc. Natl. Sci. USA, 80,

726-730, 1983). In addition, GENETYX (Software

5   Development Co., Ltd.) or the like is commercial

general- purpose software and is easily utilizable.

          The variants of the 5.9 kDa protein, 7.8 kDa

protein and 28 kDa protein, which are the marker

proteins for diagnosing liver disease of the present

10   invention, may also be variants which are proteins

having an amino acid sequence formed by deletion,

substitution or addition of one or more amino acid

residues in each of the amino acid sequences shown as

SEQ ID NOs: 1, 2 and 3 and have the same function as

15   that of the above three proteins as marker proteins for

diagnosing liver disease. As such variants, there are

exemplified proteins obtained by the modification of

less than 10%, preferably less than 5%, more preferably

less than 2%, of the amino acid residues in the

20   original amino acid sequence. The modification of the

amino acid residues may be introduced as amino acid

variation by a genetic technique generally known to

those skilled in the art. The present invention also

includes variants obtained by well-known modification

25   such as posttranslational modification,

phosphorylation, acetylation, sugar chain addition, or

the like.

          Diagnosis of liver diseases becomes possible

on the basis of the marker proteins for diagnosing
liver disease found by the present invention and
explained above.   That is, the probability of the onset
of a liver disease, the liver disease or the prognosis
5   of the liver disease may be diagnosed by detecting or
quantifying the above-mentioned marker proteins for
diagnosing liver disease in a sample obtained from a
patient who is suspected to have the liver disease.

As the sample usable in the present
10 invention, there are exemplified serum, plasma, blood
and urine collected from the patient who is suspected
to have the liver disease.

All methods known at present may be adopted
for detecting or quantifying the marker proteins for
15 diagnosing liver disease of the present invention.   The
methods include, for example, mass spectrometry method,
immunoassay method, electrophoresis method, liquid
chromatography (LC) method and gas chromatography (GC)
method.

20   As the mass spectrometry method, a method
using a laser desorption/ionization-time of flight-mass
spectrometer (LDI-TOF MS) is exemplified.   As the laser
desorption/ionization-time of flight-mass spectrometer,
there may be exemplified surface enhanced laser
25 desorption/ionization-time of flight-mass spectrometers
(SELDI-TOF MS method) and matrix-assisted laser
desorption/ionization-time of flight-mass spectrometers
(MALDI-TOF MS method).

For example, when SELDI-TOF MS method is adopted, Protein•Biology•System II•Mass•Spectrometer (Ciphergen Biosystems, Inc.) developed by Ciphergen Biosystems, Inc. may be used. This machine is based on

5   a protein chip technology comprising a combination of SELDI (surface enhanced laser desorption ionization) and a time-of-flight mass spectrometer. The details of the machine are disclosed in International Publication No. WO 01/25791 A2, JP-A-2001-28122 and the like.

10   Usually, in SELDI-TOF MS method, a sample is pretreated, adsorbed on a chip and then loaded on a SELDI-TOF MS mass spectrometer. When the sample is serum, it is preferable to remove albumin from the system by using an adsorbent for albumin or washing the

15   system with a buffer solution until the possession of electric charge by albumin owing to the ion exchange chip is ceased.

The protein chip used in such a method is not particularly limited so long as it can adsorb the

20   marker proteins for diagnosing liver disease of the present invention. As the protein chip, there may be exemplified chips (referred to also as chemical chips) in which functional groups having hydrophobicity or affinity for proteins (e.g. ion exchange properties)

25   have been modified, and chips (biochemical chips) having an antibody against the protein of interest immobilized thereon.

As another mass spectrometry method, a mass

spectrometry method using ESI method (electrospray
ionization) is exemplified. In the case of ESI method,
it is often preferable to load a sample subjected to
pretreatment such as protease treatment on a mass

5   spectrometer connected directly to a separating means
such as high performance liquid chromatography.

As the immunoassay method, there may be
exemplified an immunoassay method in which a heretofore
known protein is measured by preparing a polyclonal or

10 monoclonal antibody against any of the marker proteins
for diagnosing liver disease of the present invention.
Such an immunoassay method includes enzyme immunoassay
method (EIA method), immunoturbidimetry method (TIA
method), latex immuno-agglutination method (LATEX

15 method), electrochemiluminescence method, fluorescence
method and the like. An immuno-chromatography method
and a method using test paper are also effective. All
of these methods are generally known to those skilled
in the art and these generally known methods may be

20 adopted as they are.

As the antibody usable in the above-mentioned
immunoassay method, there are exemplified polyclonal or
monoclonal antibodies prepared by generally used
methods. These antibodies may be obtained by using a

25 purified protein derived from human blood,
specifically, the 28 kDa protein, the 7.8 kDa protein
or the 5.9 kDa protein as an immunogen (an antigen).
Although these proteins for preparing the antibodies

may be obtained from human blood by purification, they
may be obtained also by chemical synthesis by adopting
a well-known peptide synthesis technique. Besides
these antigens, proteins produced by cultured cells may
5 also be used as an antigen. In addition, the
employment of a full-length recombinant protein
prepared by genetic engineering, its variant, or a
portion of the recombinant protein or the variant is
also a well-worn measure, and this measure may be
10 utilized.

The monoclonal antibodies are produced by
hybridomas obtained by immunizing an animal with an
immunogen such as any of the above-exemplified various
antigens, for example, the 28 kDa protein, the 7.8 kDa
15 protein and the 5.9 kDa protein, namely, the marker
proteins, and then fusing antibody-producing cells
derived from the spleen or the like with myeloma cells.

The hybridomas may be obtained by the
following method. That is, the antigen (e.g. the
20 marker protein) obtained as described above is mixed
with a well-known adjuvant such as Freund's complete or
incomplete adjuvant, aluminum hydroxide adjuvant,
pertussis adjuvant or the like to prepare an adjuvant
liquid for sensitization, and this liquid is
25 administered to an animal (e.g. a mouse or a rat)
subcutaneously in the abdominal cavity or intravenously
in the tail, in several portions at intervals of 1 to 3
weeks to immunize the animal. Although the amount of

the antigen for the sensitization is usually chosen in
the range of 1 μg to 100 mg, it is preferably about 50
μg in general. Although the number of immunizing
operations is generally 2 to 7, various methods are
5 known. Subsequently, antibody-producing cells derived
from the spleen or the like are fused with myeloma
cells or the like in a test tube. As to a method for
the fusion, the fusion may be carried out by the use of
a poly(ethylene glycol) (PEG) by the method of KÖhller
10 and Milstein (Nature, 256, 495, 1975) which is already
per se well known. The fusion may be carried out also
by the use of Sendai virus or by an electrofusion
method.

As to a method for selecting hybridoma
15 capable of producing an antibody capable of recognizing
the marker protein, from the fused cells, the selection
may be carried out as follows. That is, the hybridoma
is selected from colonies formed by cells surviving in
HAT medium and HT medium in limiting dilution of the
20 fused cells. When an antibody against the marker
protein is contained in the supernatant of the culture
medium for any of the colonies formed by the fused
cells seeded into a 96-well plate or the like, a clone
capable of producing a monoclonal antibody against the
25 marker protein may be selected by an ELISA method in
which the supernatant is placed on an assay plate
having the marker protein immobilized thereon, and
after the reaction, a secondary labeled antibody such

as anti-mouse immunoglobulin- HRP labeled antibody is reacted with the above-mentioned antibody. As the labeling substance of the labeled antibody, there may be used enzymes (e.g. alkaline phosphatase),

5   fluorescent substances, radioactive substances and the like besides HRP. Screening of specific antibodies against the marker proteins, respectively, may be conducted by carrying out, as a control, ELISA using an assay plate having only BSA bonded thereto as a

10 blocking agent, simultaneously with the above-mentioned ELISA. That is, a clone is selected which is positive in any of plates having the marker proteins, respectively, immobilized thereon, and is negative in the ELISA method using BSA.

15   For example, hybridomas CN-1 and CN-2 established by the present inventor are clones capable of recognizing human 5.9 kDa protein specifically and are preferable examples. Hybridomas CN-1 and CN-2 were deposited as follows in Patented Organism Deposition

20 Center (IPOD), Industrial Technology General Research Institute (Independent Administrative Corporation), Chuo-dairoku, Higashi 1-1-1, Tsukuba City, Ibaraki Prefecture, Japan 305-8566: hybridoma CN-1 was deposited as a receipt number IPOD FERM BP-08564 on

25 Dec. 12, 2003 and hybridoma CN-2 was deposited as a receipt number IPOD FERM BP-08565 on Dec. 12, 2003.

          The hybridoma is cultured on a medium usually used for cell culture, such as α-MEM, RPMI1640, ASF, S-

clone or the like, and the monoclonal antibody may be
recovered from the supernatant of the medium.   The
following is also possible: after a nude mouse, an
animal from which the hybridoma is derived, is

5   previously treated with pristane, cells are
intraperitoneally injected into the animal to cause
accumulation of ascites, and the monoclonal antibody is
recovered from the ascites.   As a method for recovering
the monoclonal antibody from the supernatant or the

10   ascites, a conventional method may be adopted.   There
are exemplified salting-out with ammonium sulfate,
sodium sulfate or the like, chromatography, ion
exchange chromatography, and affinity chromatography
using protein A.

15   The 28 kDa protein, 7.8 kDa protein or 5.9
kDa protein in a sample may be accurately measured by
the use of the monoclonal antibody according to the
present invention purified by the above method.   As a
method for measuring the 28 kDa protein, 7.8 kDa

20   protein or 5.9 kDa protein in a sample by an EIA
method, there may be adopted a per se well known method
using one or more monoclonal antibodies against the
marker protein.   An example of the method is described
below.   At first, the monoclonal antibody (antibodies)

25   against the marker protein is directly or indirectly
bonded to a per se well-known solid phase (e.g. a
polystyrene, polypropylene, polycarbonate,
polyethylene, nylon or polymethacrylate) by utilizing

physical bonding, chemical bonding or affinity. The

amount of the sensitizing antibody ranges usually from

1 ng to 100 mg/ml. A sample is added to the monoclonal

antibody (antibodies) bonded to the solid phase by

5   physical bonding, chemical bonding or affinity to carry

out the reaction. After a definite period of the

reaction, the solid phase is washed and a corresponding

secondary labeled antibody (e.g. an anti-28 kDa protein

secondary labeled antibody, an anti-7.8 kDa protein

10   secondary labeled antibody or an anti-5.9 kDa protein

secondary labeled antibody) is added to carry out the

secondary reaction. The solid phase is washed again

and DAB color-producing substrate or the like is added

thereto to carry out the reaction. When HRP is used as

15   the labeling substance, a known substrate such as DAB,

TMB or the like may be used. The labeling substance is

not limited to HRP. As the labeling substance, there

are exemplified all recognizable substances including

not only enzymes but also labeling metals (e.g. gold

20   colloid and europium), various chemical or biological

fluorescent substances (e.g. FITC, Rhodamine, Texas

Red, Alexa and GFP) and radioactive substances (e.g. ^{32}P

and ^{51}Cr).

The marker proteins may be measured also by a

25   method other than the above-mentioned immunoassay

method, such as an electrophoresis method, liquid

chromatography (LC) method, gas chromatography (GC)

method or the like. These methods are also generally

known to those skilled in the art and these generally

known methods may be adopted as they are.

By the method explained above, the

probability of the onset of a liver disease, the liver

5   disease or the prognosis of the liver disease may be

diagnosed by detecting or quantifying the marker

proteins for diagnosing liver disease in a sample

obtained from a patient who is suspected to have the

liver disease. When the diagnosis method of the

10   present invention is practiced by the above-mentioned

mass spectrometry, the diagnosis may be carried out

also by analyzing the pattern of a spectrum obtained

with a mass spectrometer. The diagnosis method of the

present invention permits diagnosis of the probability

15   of the onset of a liver disease in a habitual drinker

or a problem drinker, diagnosis of a liver disease

caused by drinking, such as hepatitis, liver cirrhosis

or the like, and diagnosis of a usual liver disease.

Furthermore, it also permits diagnosis of, for example,

20   the progress of treatment of liver disease. The

diagnosis method of the present invention is

particularly suitable for diagnosis of alcoholic liver

troubles, alcohol dependence and the like.

The present invention is illustrated in

25   further detail with reference to the following

examples, which should not be construed as limiting the

scope of the invention.

Example 1

<u>Identification of a marker protein for</u>

<u>diagnosing liver disease by the use of SAXII protein</u>

<u>chip arrays</u>

5   Using the sera of patients from whom informed

consent had been obtained, a novel marker for liver

trouble in the sera was searched for by the use of

SAXII protein chip arrays (Ciphergen Biosystems, Inc.).

The SAXII chip refers to a strong anion exchange chip

10   and is characterized in that it binds thereto a

negatively charged substance in a sample. The sera of

the patients with alcoholic liver trouble immediately

after admission, those 1 week after abstinence and

those 3 months after abstinence and the sera of normal

15   persons were used as samples.

(1)   Method

        A method for experimental operation of the

protein chip array is briefly described below. Each

serum sample was diluted 10-fold with a 8M urea

20   (SIGMA)/1% CHAPS (SIGMA) solution. After on-ice

treatment for 10 minutes, the serum sample was further

diluted 10-fold with 50 mM Tris (SIGMA) buffer (pH 9.0)

and centrifuged at 4,000 rpm for 5 minutes, and the

supernatant was used as a diluted sample. An

25   experiment was carried out by attaching the SAXII chip

to a bioprocessor. The bioprocessor is a perforated

plastic adaptor for simply forming three-dimensional

wells on a metal chip. By such a method, a large

volume of the diluted sample may be applied.

At first, 150 μL of 50 mM Tris buffer (pH 9.0) was added to the chip having wells formed thereon, and the chip was washed on a shaker for 5 minutes.

5   After this procedure was carried out twice, 100 μL of the diluted sample previously obtained was added to the chip and shaken at room temperature for 20 minutes to be reacted with the chip. Then, the diluted sample was removed, and 150 μL of 50 mM Tris buffer (pH 9.0) was

10 added to the chip, followed by washing on a shaker for 5 minutes. This procedure was repeated three times. Thereafter, the chip was washed twice with 400 μL of distilled water and then removed from the bioprocessor. After the chip dried, each spot having a protein

15 adhered thereto was surrounded with PAPen (Zymed) and 0.5 μL of a saturated solution of sinapinic acid (Ciphergen Biosystems, Inc.) in 50% acetonitrile (Wako) and 0.5% TFA (Wako) was added twice to the spot. The protein chip arrays thus prepared were read with

20 Protein•Biology•System II•Mass•Spectrometer (Ciphergen Biosystems, Inc.).

(2)   Results

Fig. 1 shows typical measurement data obtained from the serum of a patient with alcoholic

25 liver trouble at the time of admission. In this data format, the axis of abscissa may refer to the molecular weight of a protein in each sample detached from the SAXII protein chip, and the axis or ordinate may refer

to a peak reflecting the amount of an analyte which has

arrived at the detector at the aforesaid molecular

weight. Therefore, it was found that as is clear from

Fig. 1, a peak due to a protein having a molecular

5   weight of 28 kDa was observed in the data for the

patient with alcoholic liver trouble at the time of

admission but was hardly observed after the admission.

Accordingly, it was found that a liver disease may be

diagnosed by using this 28 kDa protein as an

10   indication.

Example 2

Identification of marker proteins for

diagnosing liver disease by the use of WCXII protein

15   chip arrays

Next, using exactly the same samples as in

Example 1, novel markers for liver trouble in the sera

were searched for by the use of WCXII protein chip

arrays (Ciphergen Biosystems, Inc.). The WCXII chip

20   refers to a weak cation exchange chip and is

characterized in that it binds thereto a positively

charged substance in a sample.

(1) Method

A method for experimental operation of the

25   protein chip array is briefly described below and is

substantially the same as in Example 1. Each serum

sample was diluted 10-fold with a 8M urea (SIGMA)/1%

CHAPS (SIGMA) solution. After on-ice treatment for 10

minutes, the serum sample was further diluted 10-fold with 50 mM sodium acetate (SIGMA) buffer (pH 6.5) and centrifuged at 4,000 rpm for 5 minutes, and the supernatant was used as a diluted sample. An

5 experiment was carried out by attaching the WCXII chip to a bioprocessor. At first, 150 µL of 50 mM sodium phosphate buffer (pH 6.5) was added to the chip having wells formed thereon, and the chip was washed on a shaker for 5 minutes. After this procedure was carried

10 out twice, 100 µL of the diluted sample previously obtained was added to the chip and shaken at room temperature for 20 minutes to be reacted with the chip. Then, the diluted sample was removed, and 150 µL of 50 mM sodium phosphate buffer (pH 6.5) was added to the

15 chip, followed by washing on a shaker for 5 minutes. This procedure was repeated three times. Thereafter, the chip was washed twice with 400 µL of distilled water and then removed from the bioprocessor. After the chip dried, each spot having a protein adhered

20 thereto was surrounded with PAPen (Zymed) and 0.5 µL of a saturated solution of sinapinic acid (Ciphergen Biosystems, Inc.) in 50% acetonitrile (Wako) and 0.5% TFA (Wako) was added twice to the spot. The protein chip arrays thus prepared were read with

25 Protein•Biology•System II•Mass•Spectrometer (Ciphergen Biosystems, Inc.).

(2) Results

Fig. 2 shows typical measurement data

obtained from the serum of a patient with alcoholic

liver trouble at the time of admission. Fig. 3 shows

measurement data for normal persons. The following was

found: as is clear from Fig. 3, the peaks at a

5 molecular weight of 5,900 Da (the 5.9 kDa protein) and

a molecular weight of 7,800 Da (the 7.8 kDa protein)

are high in the data for the normal persons, but these

peaks are hardly observed in the data shown in Fig. 2,

i.e., the data for the patient with alcoholic liver

10 trouble immediately after admission. The heights of

the peaks due to the 5.9 kDa protein and the 7.8 kDa

protein increase with treatment and clearly indicate

the effect of the treatment. It was found that these

proteins may be used as marker proteins for diagnosing

15 liver disease.

Example 3

Identification of the 28 kDa protein

(1) The 28 kDa protein found by the SAXII

20 protein chip experiment was purified from a serum

sample with FPLC Pharmacia LKB (Amersham Pharmacia

Biotech AB) by the use of a HiTrap Q column under the

following conditions: 50 mM Tris buffer (pH 9.0) and a

flow rate of 2 ml/min. Thus, the 28 kDa protein of

25 interest could be purified as a substantially single

fraction. This fraction was confirmed as follows by

electrophoresis. The fraction was mixed with 2 x

sample buffer (0.25 M Tris-HCl (pH 6.8), 4% SDS, 20%

glycerol, 0.01% BPB and 10% β-mercaptoethanol) in the
ratio of 1: 1 and treated at 90°C for 2 minutes. The
fraction thus treated was used in the electrophoresis.
The electrophoresis was carried out at 10 mA by the use
5   of 15 to 25% polyacrylamide gradient gel (Perfect NT
Gel System Products).

         (2)   As shown in each of the lanes 4 and 5 in
Fig. 4, a band was confirmed at a position
corresponding to 28 kDa, by Coomassie Brilliant Blue
10   staining using Coomasie Tablet R-350 (Phast GI Blue R).

         (3)   Then, the band of the gel was excised
and peptides were separated by an In-Gel digestion
method. In brief, the piece of the gel obtained by
excision was washed twice and then treated overnight
15   with trypsin at 35°C. Thereafter, the sample treated
with trypsin was purified by reversed phase HPLC. As
to the purification conditions, gradient elution with
0.1% TFA and 0.09% TFA 90% acetonitrile was carried out
by the use of a TSK gel ODS-80Ts QA (TOSOH) column.

20   The internal amino acid sequences of the
resulting 28 kDa protein fragments were determined.
The amino acid sequence of the 28 kDa protein is shown
as SEQ ID NO: 3 in the sequence listing. As the result
of amino acid sequencing of the 28 kDa protein
25   fragments, the 28 kDa protein was found to be human
apolipoprotein AI on the basis of the internal amino
acid sequences of the 28 kDa protein fragments.

         (4)   Then, apolipoprotein AI levels were

measured for the same serum samples as used in the chip
experiments by an immunoassay method using the known
autoanalyzer described in Example 6, to obtain the
results shown in Table 1. From the immunoassay

5   results, it was found that apolipoprotein AI levels
were clearly reduced after treatment in the samples
obtained from the patients who had gained effect from
the treatment. On the other hand, the height of the
peak due to the 28 kDa protein (apolipoprotein AI)

10   observed as a result of the protein chip experiment was
also reduced by the treatment, namely, it reflected the
effect of the treatment. Thus, it was found that there
is a very high correlation between the result obtained
by the immunoassay method and the result of the protein

15   chip experiment, indicating that the height of the peak
is utilizable for analyzing morbidity.

Example 4

        Identification of the 7.8 kDa protein

20   (1)   Like the 28 kDa protein, the 7.8 kDa
protein found by the WCXII protein chip experiment was
also purified from a serum sample with FPLC Pharmacia
LKB (Amersham Pharmacia Biotech AB) by the use of a
HiTrap CM Sepharose FF column under the following

25   conditions: 50 mM ammonium acetate buffer and a flow
rate of 2 ml/min. Thus, the 7.8 kDa protein of
interest could be purified. A fraction in which the
7.8 kDa protein had been confirmed by the protein chip

experiment was confirmed by electrophoresis as follows.

The fraction was mixed with 2 x sample buffer

containing SDS, in the ratio of 1 : 1 and treated at

90°C for 2 minutes. The fraction thus treated was used

5   in the electrophoresis. The electrophoresis was

carried out at 10 mA by the use of 15 to 25%

polyacrylamide gradient gel.

(2)   As shown in each of the lanes 2 and 3 in

Fig. 4, a band was confirmed at a position

10   corresponding to 7.8 kDa, by Coomassie Brilliant Blue

staining. As in Example 3 for the above-mentioned 28

kDa protein, a fraction containing substantially only

this protein of 7.8 kDa was concentrated and then

subjected to SDS-PAGE, and the 7.8 kDa band of interest

15   was excised from the gel and peptides were separated by

an In-Gel digestion method. This method is the same as

in Example 3. In brief, the piece of the gel obtained

by excision was washed twice and then treated overnight

with trypsin at 35°C. Thereafter, the sample treated

20   with trypsin was purified by reversed phase HPLC. As

to the purification conditions, gradient elution with

0.1% TFA and 0.09% TFA 90% acetonitrile was carried out

by the use of a TSK gel ODS-80Ts QA (TOSOH) column.

(3)   The resulting 7.8 kDa protein fragments

25   were subjected to amino acid sequencing and found to be

derived from human apolipoprotein AI, on the basis of

the internal amino acid sequences of the 7.8 kDa

protein fragments. However, the theoretical molecular

weight of full-length human apolipoprotein AI is 11432.4. That is, the 7.8 kDa protein was found to be a human apolipoprotein AI decomposition product. The amino acid sequence of the 7.8 kDa protein is shown as

5   SEQ ID NO: 2.

Example 5

Identification of the 5.9 kDa protein

(1)   The 5.9 kDa protein found by the WCXII

10   protein chip experiment was purified from a serum sample with FPLC Pharmacia LKB (Amersham Pharmacia Biotech AB) by the use of a HiTrap CM Sepharose FF column under the following conditions: 50 mM ammonium acetate buffer and a flow rate of 2 ml/min. Thus, the

15   5.9 kDa protein of interest could be purified. A fraction having a high content of the 5.9 kDa protein was checked again by a protein chip method, concentrated and then further purified by HPLC (TOSOH). The purification conditions were as follows: a Sephasil

20   protein C4 column (Amersham Pharmacia Biotech AB), an acetonitrile gradient, and 1 mL/min.

(2)   The fraction was checked again by a protein chip method, concentrated by freeze-drying and then subjected to amino acid sequencing. As a result

25   of the amino acid sequencing of the purified 5.9 kDa protein, this protein was found to be a human fibrinogen α-E chain decomposition product. That is, since the molecular weight of full-length human

fibrinogen α-E chain is 72488.3, the 5.9 kDa protein was found to be a human fibrinogen α-E chain decomposition product. The amino acid sequence of the 5.9 kDa protein is shown as SEQ ID NO: 1.

5 In addition, in order to confirm the accuracy of the amino acid sequence of the 5.9 kDa protein, a protein having this amino acid sequence was wholly and chemically synthesized. The molecular weight of the chemically synthesized protein having 54 amino acids
10 was 5904.1 which agreed with the theoretical value (Fig. 5). Furthermore, this synthetic protein was compared with an actual sample. An experiment was carried out by the use of exactly the same WCXII protein chip arrays (Ciphergen Biosystems, Inc.) as in
15 Example 2. In the experiment, the synthetic protein was reacted in a concentration of 100 ng/mL as a sample and compared with the actual sample. The results are shown in Fig. 6. As a result, a peak due to the synthetic protein showed a behavior coinciding with a
20 peak due to the 5.9 kDa protein in the serum sample. By these results, it was confirmed that the 5.9 kDa protein in the serum had the amino acid sequence shown as SEQ ID NO: 1.

25 Example 6

Comparison between diagnosis in patients with alcoholic liver trouble based on the marker proteins for diagnosing liver disease of the present invention

and that based on conventional markers for hepatitis

(1)   Method

Measured values for conventional markers for hepatitis were obtained by using the same sera as used

5   above, i.e., the sera of the 16 inpatients with alcoholic liver trouble immediately after admission, those after 1 week of hospitalization and those after 3 months of hospitalization. Measurement with an autoanalyzer was carried out as follows. AST, GGT, TG,

10   Apo AI and Apo AII were measured with Hitachi 7150 analyzer (HITACHI). As reagents, N-assay L GOT (AST) (Nittobo), N-assay L γ-GTP-H (GGT) (Nittobo), N-assay TG L (TG) (Nittobo), N-assay TIA Apo AII-H (Apo AI) (Nittobo) and N-assay TIA Apo AII (Apo AII) (Nittobo)

15   were individually used. FDP and FDP-E were measured with LPIA-S500 (Diayatron) by using predetermined parameters. In this measurement, LPIA FDP latex and LPIA FDP-E latex (Teikoku Hormone MFG. Co., Ltd.) were used as reagents. For comparison, the results of

20   measurement using protein chips are expressed by numeral values on the basis of peaks. The units of the numeral values are arbitrary units.

(2)   Results

Table 1 shows the following items in the

25   common sera of patients hospitalized with alcoholic liver trouble: the levels of biochemical measurement markers for clinical examination (AST, GGT and TG), the levels of immunological measurement markers (Apo AI,

Apo AII, FDP and FDP-E) and the results of measurement of the marker proteins for liver disease of the present invention by a protein chip method. In the first and second columns (the "FDP-E 5,900 Da" column and the

5   "Apo AII 7,800 Da" column) from the right of Table 1, there are shown measured values obtained by measuring the 5.9 kDa protein and the 7.8 kDa protein, i.e., the marker proteins for diagnosing liver disease of the present invention, by a method using the same protein

10 chips as in Example 2. In the sixth column (the "Apo AI" column) from the right of Table 1, there are shown measured values obtained by measuring the 28 kDa protein, i.e., the marker protein for diagnosing liver disease of the present invention, by a conventional

15 method.

Table 2 shows measured values obtained by measuring the 5.9 kDa protein (FDP-E 5,900 Da) and the 7.8 kDa protein (Apo AII 7,800 Da), i.e., the marker proteins for diagnosing liver disease of the present

20 invention, in serum samples from healthy persons.

Table 3 shows average measured values obtained by measuring the 5.9 kDa protein and the 7.8 kDa protein in the inpatients with alcoholic liver disease by a protein chip method.

25

Table 1

Changes of clinical examination values in patients with alcoholic liver trouble after abstinence

		AST U/L	GGT U/L	TG mg/dL	Apo AI mg/dL	Apo AII mg/dL	FDP µg/mL	FDP-E ng/mL	FDP-E 5,900Da AU	Apo AII 7,800Da AU
No. 1	On admission	34	542	672	144	37.3	2.99	110.32	11.7	9.9
	After hospitalized for 1 week	39	559	246	121	27.8	2.76	135.69	24.2	10.9
	After hospitalized for 3 months	25	121	142	79	17.4	1.63	80.20	20.8	6.1
No. 2	On admission	13	9	103	88	15.0	3.57	77.28	0	3.1
	After hospitalized for 1 week	30	16	98	94	15.1	3.69	136.11	1.0	11.7
	After hospitalized for 3 months	15	3	173	95	20.8	2.65	126.56	1.8	20.0
No. 3	On admission	42	68	100	155	38.2	1.37	70.18	0	0
	After hospitalized for 1 week	22	45	91	105	26.0	7.05	592.26	0	1.7
	After hospitalized for 3 months	18	19	117	104	21.5	15.96	1257.50	0.7	5.0
No. 4	On admission	86	1452	150	234	45.0	3.39	93.30	0.5	7.9
	After hospitalized for 1 week	21	701	73	112	26.1	5.24	347.94	3.5	12.6
	After hospitalized for 3 months	15	24	68	95	17.7	7.27	516.60	17.7	21.1
No. 5	On admission	24	74	93	95	21.8	0.31	35.11	0.8	2.4
	After hospitalized for 1 week	23	64	139	86	21.0	1.36	47.86	4.8	16.1
	After hospitalized for 3 months	35	160	154	80	18.5	3.27	71.87	6.2	18.1
No. 6	On admission	44	108	141	149	37.7	1.56	42.53	9.2	12.1
	After hospitalized for 1 week	25	64	117	98	26.0	3.56	248.95	48.0	21.4
	After hospitalized for 3 months	17	24	109	83	19.3	8.88	743.54	58.0	20.0
No. 7	On admission	37	272	119	218	46.5	2.54	90.77	0	0
	After hospitalized for 1 week	27	174	77	157	33.7	2.05	91.00	23.0	18.4
	After hospitalized for 3 months	19	26	81	95	23.3	1.58	50.95	21.7	10.4
No. 8	On admission	40	61	170	183	33.3	3.87	127.00	1.7	3.3
	After hospitalized for 1 week	24	57	65	113	22.7	3.17	189.23	0	5.7
	After hospitalized for 3 months	22	36	113	101	20.6	2.35	105.10	1.8	6.9
No. 9	On admission	20	77	244	114	28.4	4.33	118.19	0	0.4
	After hospitalized for 1 week	25	65	121	109	24.9	50.13	330.58	0	7.1
	After hospitalized for 3 months	24	36	260	109	24.0	5.33	270.77	1.7	14.0
No. 10	On admission								2.0	5.6
	After hospitalized for 1 week	55	232	67	69	18.5	13.52	915.69	8.7	16.1
	After hospitalized for 3 months	17	41	54	107	20.6	0.87	35.35	1.7	21.4
No. 11	On admission	38	81	187	151	34.5	2.82	58.02	15.8	19.3
	After hospitalized for 1 week	20	67	98	116	28.1	6.33	554.17	56.0	26.4
	After hospitalized for 3 months	22	18	74	104	18.1	15.51	1310.60	52.0	18.6
No. 12	On admission	18	37	103	135	24.8	1.91	43.10	0	11.7
	After hospitalized for 1 week	17	31	110	126	19.6	1.78	68.85	0.8	6.4
	After hospitalized for 3 months	21	32	118	136	22.6	1.03	33.59	1.2	15.0
No. 13	On admission	18	31	84	198	30.6	1.99	38.14	12.2	9.4
	After hospitalized for 1 week	100	23	72	154	22.7	6.74	342.92	32.8	13.9
	After hospitalized for 3 months	17	18	80	180	22.6	14.37	1118.50	45.0	15.0
No. 14	On admission	63	44	333	154	39.5	3.07	96.96	0	3.1
	After hospitalized for 1 week	43	39	72	111	27.0	2.50	146.95	0.3	11.0
	After hospitalized for 3 months	37	26	159	92	23.2	4.73	382.86	0.3	12.1
No. 15	On admission	49	86	91	146	28.9	1.35	58.93	13.0	7.0
	After hospitalized for 1 week	19	79	82	83	23.0	2.29	59.44	28.5	17.7
	After hospitalized for 3 months	22	60	112	86	22.5	2.99	177.59	51.0	18.0
No. 16	On admission	29	84	90	162	31.3	3.24	88.54	2.5	1.3
	After hospitalized for 1 week	12	70	118	102	26.6	1.86	84.94	1.7	10.3
	After hospitalized for 3 months	16	15	96	72	17.0	5.84	213.87	2.0	13.3

Table 2

Levels of 5.9 kDa and 7.8 kDa proteins in
healthy persons

Number of healthy persons (n=12)	FDP-E 5.9kDa AU	Apo A II 7.8kDa AU
No.1	59.1	35.1
No.2	51.7	32.0
No.3	62.5	42.8
No.4	63.1	44.0
No.5	89.2	95.4
No.6	58.5	42.2
No.7	64.6	45.5
No.8	66.2	44.6
No.9	23.1	56.9
No.10	55.4	51.7
No.11	63.7	52.0
No.12	36.0	24.6

5   Table 3

Average levels of 5.9 kDa and 7.8 kDa proteins

	FDP-E 5.9kDa AU	Apo A II 7.8kDa AU
Healthy persons(n=12)	57.76	47.23
Immediately after admission	4.34	6.03
After hospitalized for 1 week	14.58	13.00
After hospitalized for 3 months	17.73	15.63

Number of patients (n = 16)

As shown in Tables 1 to 3, the decrease of

10   generally used AST and GGT levels and the recovery of

these levels to normal level ranges reflected the

effect of treatment on liver. However, the blood

levels of the 5.9 kDa protein and the 7.8 kDa protein

increased and clearly indicated the effect of treatment

15   even when the generally used GGT level was not usable

as an indication of the effect of treatment as in the
case of sample No. 5 from a patient who was considered
as a nonresponder. The GGT level does not always
correlate with the degree of seriousness of a liver

5 trouble or the cumulative alcohol intake. In addition,
the change of the GGT level after alcohol drinking
varies depending on individuals and there are a
considerable number of so-called nonresponders who show
no increase in the GGT level even after drinking a

10 large volume of alcohol. Therefore, the novel proteins
were considered to be effective in judging the
treatment of these GGT nonresponders.

Example 7

15   Correlation between alcohol intake and the
marker proteins of the present invention

By the working examples described above, the
usefulness of the novel markers for diagnosing liver
disease found by the present invention was confirmed

20 with respect to their specificity. The following
experiment was carried out for further elucidation of
the correlation between alcohol intake and the markers.
Method

An experiment was carried out by collecting

25 samples only from healthy persons and alcohol-drinking
patients whose alcohol intake was certain. In the
experiment, these subjects were divided into three
experimental groups, i.e., a group of healthy persons

as nondrinkers, a group of patients having an alcohol intake corresponding to 1 go of sake and a group of patients having an alcohol intake corresponding to 3 go of sake. For these groups, measurements using protein

5 chip methods, respectively, were carried out and the groups were compared with respect to each of the novel markers. The measuring methods using protein chips were exactly the same as described in the case of SAXII protein chip arrays in Example 1 and WCXII protein chip

10 arrays in Example 2.

Results

The results are shown in Fig. 7. As a result, it was found that the heights of peaks due to all the novel marker proteins, respectively, for

15 diagnosing liver disease vary depending on alcohol intake. Here, the results for, in particular, the 5.9 kDa protein are shown. The 5.9 kDa protein decreased depending on alcohol intake and it decreased to become substantially undetectable in the experimental group

20 having an alcohol intake of 3 go. That is, it was found that the 5.9 kDa protein varies in amount, depending on alcohol intake and hence is a marker satisfactorily utilizable for estimation of alcohol intake. It was also found that the 5.9 kDa protein is

25 utilizable as a marker for alcohol dependence.

Example 8

Preparation of a monoclonal antibody

The following experiment was carried out for preparing an anti-5.9 kDa protein monoclonal antibody by using the completely synthetic 5.9 kDa protein obtained in Example 5, as an antigen.

5 (1) Immunization

The completely synthetic 5.9 kDa protein was diluted to a concentration of 1 mg/ml with phosphate buffer (pH 7.0), and 50 µg (50 µl) of the dilution was thoroughly mixed with 50 µl of Freund's complete

10 adjuvant (WAKO) until emulsification was effected. The suspension thus prepared was intraperitoneally administered to a Balb/c female mouse aged 6 weeks (Nippon Clear Co., Ltd.) under anesthesia with diethyl ether. After 2 weeks, the same amount as above of the

15 completely synthetic 5.9 kDa protein (50 µg/ml) was mixed with Freund's incomplete adjuvant (WAKO). By exactly the same procedure as in the case of the Freund's complete adjuvant, emulsification was effected to obtain a suspension and the mouse was sensitized

20 with the suspension. Two weeks after the sensitization, the same procedure as above was carried out. For the fourth immunization, i.e., final immunization, a dilution of the completely synthetic 5.9 kDa protein (50 µg/ml) with phosphate buffer (pH

25 7.0) was prepared and then administered to the mouse by injection into the tail vein.

(2) Establishment of hybridoma

Three days after the final immunization, the

spleen was surgically removed from the mouse sensitized

with the synthetic 5.9 kDa protein, under anesthesia

with diethyl ether, and was aseptically dispersed to

prepare splenocytes. Fusion was carried out according

5   to the method of Köhller and Milstein (Nature, 256,

495, 1975). The splenocytes were fused with myeloma

cells P3-X63-Ag8-U1 (P3U1) by the use of a

poly(ethylene glycol) (PEG4000) (MERK). As to the

fusion ratio, the number of the splenocytes was 10 x

10   10^7, while the number of the myeloma cells P3-X63-Ag8-U1

(P3U1) was 2 x 10^7. That is, the fusion ratio of the

splenocytes to the myeloma cells was 5 : 1. The fused

cells were dispersed in 10% FCS (INVITROGEN) α-MEM

(IRVINE) HAT (Cosmo Bio Co., Ltd.), seeded into a 96-

15   wells microtiter culture plate (Sumitomo Bakelite Co.,

Ltd.) and then cultured under conditions of 37°C and 5%

CO_2.

(3)   Screening

After about 2 weeks, the growth of colonies

20   was confirmed and screening was conducted. A method

for conducting the screening is described below. For

producing a plate for the screening, the synthetic 5.9

kDa protein purified in the above working example was

dissolved in phosphate buffer and fed into a 96-well

25   plate (Nunc) in an amount of 1 μg/100 μl/well. The

plate was allowed to stand at 4°C for two nights and

then washed three times with phosphate buffer

containing 0.05% Tween 20. Each well was fed with 200

μl of 1.5% BSA solution in order to inhibit a
nonspecific reaction, and the plate was allowed to
stand overnight at 4°C. After the thus completed plate
was washed three times with phosphate buffer containing

5   0.05% Tween 20, 100 μl of the culture supernatant was
reacted in each well and the plate was further washed.
Then, HRP-labeled anti-mouse immunoglobulin antibody
(Zymed), a secondary antibody was added to carry out
the reaction. After washing, 100 μl of a 3 mg/ml

10 color-producing solution of o-phenylenediamine (OPD)
(Nacalai tesque), a color-producing substrate for HRP,
in citric acid was added to each well to cause
coloration for a definite period. Then, 100 μl of 1N
sulfuric acid was added to each well as a terminating

15 solution and absorbance was measured at a measuring
wavelength of 492 nm. Clones found to be positive by
the above procedure were subjected to recloning by a
limiting dilution method, and the resulting
supernatants were checked again.

20 (4) Confirmation of antibodies

        Two clones, i.e., clones CN-1 and CN-2 were
selected as clones which had recognized the synthetic
5.9 kDa protein, by confirming their reactivity with
the synthetic 5.9 kDa protein by ELISA. Table 4 shows

25 the results of assaying antibodies produced by these
clones, by the use of a monoclonal antibody typing kit
(Amersham Pharmacia Biotech).

Table 4 : Characteristics of monoclonal antibodies

Hybridoma	Class	Light chain
Clone CN-1	IgM	κ
Clone CN-2	IgM	κ

(5)　Preparation and purification of the monoclonal antibodies

5　　　To a Balb/c female mouse aged 10 weeks (Nippon Clear Co., Ltd.) two weeks after administration of 0.5 ml of pristane (Aldrich) to the mouse were intraperitoneally administered 1×10^7 cells of each of the hybridomas CN-1 and CN-2 obtained. After about 2 10 weeks, ascites accumulated in the abdominal cavity of the mouse was surgically collected under anesthesia with diethyl ether. As a result of confirmation by the ELISA method adopted in the screening, by the use of the ascites stepwise diluted as a sample, it was found 15 that the ascites contained a high concentration of the monoclonal antibody. The ascites was treated with 40% ammonium sulfate and dialyzed against PBS, and then the monoclonal antibodies CN-1 and CN-2 were purified by the use of S-300. As a result, a single band was 20 confirmed at a molecular weight of about 900,000 when each of the monoclonal antibodies CN-1 and CN-2 had not been reduced, and two bands were confirmed at molecular weights of about 70,000 and 25,000 when each of them had been reduced with mercaptoethanol. The amount of 25 each of the purified antibodies CN-1 and CN-2 was about

10 mg or more per mouse, namely, it was sufficient for industrial utilization.

Example 9

5   Measurement of the 5.9 kDa protein by EIA method

There was investigated the possibility of measurement of the 5.9 kDa protein in a sample by ELISA method (EIA method) by the use of the two monoclonal

10   antibodies CN-2 and CN-1 against the 5.9 kDa protein as a primary antibody and a secondary antibody, respectively.

(1) Method

For preparing a plate for ELISA, the primary

15   antibody CN-2 was dissolved in phosphate buffer (pH 6.7) and fed into a 96-well plate (Nunc) in an amount of 1 µg/100 µl/well. The plate was allowed to stand at 4°C for two nights and then washed three times with phosphate buffer containing 0.05% Tween 20. Each well

20   was fed with 200 µl of 1.5% BSA solution in order to inhibit a nonspecific reaction, and the plate was allowed to stand overnight at 4°C. After the thus completed plate was washed three times with phosphate buffer containing 0.05% Tween 20, 100 µl of various

25   concentrations of the synthetic 5.9 kDa protein was added to each well as a reference standard and the reaction was carried out at room temperature for 1 hour. After completion of the reaction, the plate was

washed three times with phosphate buffer containing

0.05% Tween 20, and HRP-labeled CN-1 antibody as

secondary antibody was added to carry out the reaction.

After washing, 100 µl of a 3 mg/ml color-producing

5 solution of o-phenylenediamine (OPD) (Nacalai tesque),

a color-producing substrate for HRP, in citric acid was

added to each well to cause coloration for a definite

period. Then, 100 µl of 1N sulfuric acid was added to

each well as a terminating solution and absorbance was

10 measured at a measuring wavelength of 492 nm.

(2)   Results

On the basis of coloration values measured

for the various concentrations of the synthetic 5.9 kDa

protein as described above, a standard curve for

15 measuring the synthetic 5.9 kDa protein was prepared to

obtain the result shown in Fig. 8. As can be seen from

Fig. 8, absorbance increased depending on the

concentration of the synthetic 5.9 kDa protein. That

is, it was found that since the monoclonal antibodies

20 CN-1 and CN-2 capable of recognizing the 5.9 kDa

protein were different in epitope, the 5.9 kDa protein

could be measured by using these antibodies in sandwich

ELISA method.

25 INDUSTRIAL APPLICABILITY

As concretely described above, the marker

proteins for diagnosing liver disease of the present

invention may be used as an indication for accurately

investigating the effect of judgment for the treatment
of a patient with liver disease due to drinking to
grasp the condition of the patient, even when the GGT
level of the patient is high owing to a cause other

5   than drinking as in the case of, for example, a
nonresponder having a low reactivity with conventional
markers for hepatitis (e.g. GGT), fatty liver
accompanying corpulence, or a person who commonly uses
a certain medicine. That is, GGT has a low specificity

10 because it is increased by a cause other than drinking,
such as a viral chronic liver trouble, corpulence, or
continuous use of a certain medicine. On the other
hand, the marker proteins for diagnosing liver disease
of the present invention are advantageous in that their

15 specificity is hopeful because their levels do not vary
even in the case of viral liver cirrhosis. It is also
found that these marker proteins are characterized by
permitting easy handling of samples and giving a high
reproducibility of measurement because substances to be

20 measured in this case are peptides which are not
dependent on biochemical enzymatic function. The
diagnosis method of the present invention also permits
diagnosis of the probability of the onset of a liver
disease in a habitual drinker or a problem drinker and

25 diagnosis of a liver disease caused by drinking, such
as hepatitis, liver cirrhosis or the like. Moreover,
it also permits diagnosis of, for example, the progress
of treatment of such a liver disease. The diagnosis

method of the present invention is suitable for diagnosis of, in particular, an alcoholic liver trouble, alcohol dependence or the like. In the diagnosis method of the present invention, diagnosis

5 may be carried out by measuring the marker proteins by a generally used EIA method, immuno-chromatography, test paper or the like. The fact that the diagnosis can easily be carried out by such a generally used method is considered to be of great significance from

10 the viewpoint of future preventive medicine in consideration of the present population of liver disease sufferers.